Evidence that cell surface charge reduction modifes capillary red cell velocity–flux relationships in hamster cremaster muscle

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1. From capillary red cell velocity (V)–flux (F) relationships of hamster cremaster muscle a yield velocity ($V_{F=0}$) can be derived at which red cell flux is zero. Red cell velocity becomes intermittent and/or red blood cells come to a complete standstill for velocities close to this yield velocity, and, at the same time, capillary tube haematocrit becomes very low.

2. We have tested whether the net negative charge of red blood cells (RBCs) contributes to the magnitude of $V_{F=0}$. Velocity–flux relationships were measured for normal cells, normal cells labelled with the fluorescent dye calcein (LRBCs), and red cells treated with hexadimethrine to mask negative charge and labelled with calcein as well (HDM-LRBCs). Measurements were done in a hamster cremaster muscle preparation applying video *in vivo* microscopy.

3. Hexadimethrine treatment reduced the net negative surface charge of red cells to 20% of control as estimated from transmission electron microscopy using a ferritin tagging technique. The values of $V_{F=0}$ found for normal red cells and HDM-LRBCs were 86 ± 15 and $31 \pm 17 \ \mu\text{m s}^{-1}, \pm$ s.e.m., $n = 12$, respectively, which were significantly different ($P < 0.05$). For normal cells and cells labelled with calcein only, $V_{F=0}$ values were 63 ± 14 and $65 \pm 13 \ \mu\text{m s}^{-1}$, $n = 8$, respectively, which were not significantly different. The effect of HDM treatment did not alter filterability of the red cells as estimated from transit times through 5 μm pores.

4. The present findings demonstrate that the net negative charge of RBCs contributes significantly to the yield velocity for red blood cells entering capillaries and flowing through them. HDM treatment reduced the net negative charge of red blood cells and may have caused cells to enter capillaries more easily owing to reduced electrostatic repulsion at the capillary entrance. In addition, HDM treatment may have lowered intracapillary flow resistance by a reduction in electrostatic repulsive forces between red blood cells and negatively charged (macromolecules on) capillary endothelial cells at sites of irregular capillary cross-sectional shape, without significantly affecting the lubricating properties of the capillary endothelial glycocalyx and/or associated plasma macromolecules.

Red blood cells (RBCs) bear a net negative charge on their surface, which is mainly due to the negative charge of the carboxylate group of sialic acid and partly due to that of acidic amino acid residues in sialoglycoproteins. Recently it was demonstrated that the surface charge, and the ensuing electrostatic repulsive forces between adjacent cells, prevents aggregation of RBCs by plasma macromolecules such as fibrinogen and immunoglobulin G (Izumida, Seiyama & Maeda, 1991). Electrostatic forces may also exist between RBCs and vascular endothelial cells. Endothelial cells are coated with a glycoprotein layer, the glycocalyx, which contains negatively charged sulphate and carboxylate groups (Desjardins & Duling, 1990; Baldwin, Wu & Stein, 1991; Ihrcke, Wrenshall, Lindman & Platt, 1993). In addition, it has been reported that certain plasma macromolecules, which are negatively charged at physiological pH, may accumulate on microvascular endothelial cells *in vivo* (Witte, 1983, 1988). Thus, as well as counteracting RBC aggregation, electrostatic repulsion may prevent close contact between cells and the vascular

wall. This effect would be most pronounced at the capillary level, where RBCs are squeezed into vessels with diameters similar to red cell dimensions.

Two relatively easily measured quantities that provide information on *in vivo* capillary perfusion by red blood cells are red cell velocity (V, μm s^{-1}) and red cell flux (F, s^{-1}). It was found that capillary red cell velocity–flux relationships show a positive intercept at the velocity axis between 11 and 201 μm s^{-1}, denoted as $V_{F=0}$ (Vink, 1994). At this yield velocity, RBC flow into a capillary segment stops and RBC motion inside the capillary segment becomes intermittent or comes to a complete halt. In the present study it was hypothesized that electrostatic repulsive forces between RBCs and the capillary wall or between RBCs and negatively charged plasma proteins associated with the capillary wall contribute to this yield velocity. To test this hypothesis, capillary red cell velocity–flux relationships were determined for both normal RBCs and for RBCs with a reduced net surface charge.

Reduction of the net surface charge of RBCs was obtained by treating them with hexadimethrine (HDM) polycations. Normal and treated RBCs were observed 'simultaneously' while flowing through the same capillary segments. To distinguish the two types of cells, HDM-treated RBCs (HDM-LRBCs) were filled with a fluorescent dye. To test for an effect of the dye only on the flow behaviour of RBCs, several *in vivo* experiments were performed with fluorescent dye-labelled RBCs which did not receive HDM treatment (control LRBCs). The filterability of RBCs was determined *in vitro* to test for an effect of the dye or HDM treatment on cell deformability. Finally, the deposition of anionic sites on RBCs was visualized with transmission electron microscopy to verify whether HDM treatment had indeed reduced the net negative charge of the RBC membrane.

METHODS

Animal preparation

Male golden hamsters (age, 10–13 weeks; weight, 89–126 g; $n = 10$) were anaesthetized with sodium pentobarbitone (I.P., 70 mg (kg body weight)$^{-1}$). The left femoral vein was cannulated for continuous infusion of pentobarbitone (10 mg ml^{-1} at 0·5 ml h^{-1}) and injection of labelled cells (0·2–0·3 ml, 60% haematocrit). The hamster was placed on a clear Plexiglass plate. A glass pedestal, contained in a silicon ring, was mounted on top of the plate. The scrotum was separated from the cremaster muscle and loose connective tissue was removed. The cremaster muscle was positioned onto the pedestal and a mid-line incision from base to top was made to open it longitudinally. The testis was dissected. Small insect pins were used to secure the free edge of the cremaster muscle to the silicon surrounding the pedestal. The muscle was superfused with bicarbonate-buffered physiological

saline solution (34 °C, pH 7·35–7·45) of the following composition (mM): 131·9 NaCl, 4·7 KCl, 2·0 CaCl$_2$.2H$_2$O, 1·2 MgSO$_4$.7H$_2$O and 20·0 NaHCO$_3$. Superfusion solutions were equilibrated with gas containing 5% CO$_2$–95% N$_2$ and 45–60 min after completion of the surgery the cremaster muscle vasculature was maximally vasodilated by adding 10^{-4} M adenosine to the superfusate prior to the start of the experimental protocols.

Video microscopy

Perfusion of single capillaries with normal red blood cells was observed by transillumination of the cremaster muscle and images of the microvasculature were projected onto a television monitor at a final magnification of × 1100. Perfusion of capillaries with labelled cells was visualized by epi-illumination of the preparation with a 100 W mercury lamp through a standard blue/green (excitation/emission) dichroic mirror set. Images of fluorescent cells were displayed on the television monitor at a final magnification of × 1100 using a × 50 microscope objective (MA50 Olympus, NA = 0·70) and a CCD camera equipped with an image intensifier tube.

Labelling of red blood cells

Erythrocytes were filled with calcein dye. This fluorescent dye was entrapped in RBCs by resealing of cell ghosts produced by a modification of a pre-swell loading procedure (Nutall, 1987). Blood was obtained from anaesthetized male golden hamsters by heart puncture and was collected into 3 ml tubes containing Thromboliquine Heparine natrium (Organon Teknika) and stored at 4 °C. The plasma was separated by centrifugation, and the cells were washed three times in four times their volume of phosphate-buffered saline (PBS) of the following composition (mM): 137·0 NaCl, 2·7 KCl, 8·1 Na$_2$HPO$_4$.7H$_2$O, 1·4 NaH$_2$PO$_4$.H$_2$O and 4·0 MgSO$_4$.7H$_2$O (300 mosmol kg^{-1}, pH 7·35–7·45). Washed packed cells were suspended in four times their volume of swelling solution (150 mosmol kg^{-1}), i.e. PBS diluted 1:1 (vol:vol) with distilled H$_2$O. After 5 min this suspension was centrifuged and packed cells were again placed in the swelling solution. Thereafter, lysis was induced by adding the cells to three or four times of their volume of a lysing dye solution (100 mosmol kg^{-1}), i.e. PBS diluted 1:2 (vol:vol) with distilled H$_2$O to which calcein was added (1 mg ml^{-1}). This suspension was incubated at 40 °C for about 60 min, after a 2–4 min equilibration period. Then the cells were resealed by addition of sufficient PBS, i.e. 3–5 ml PBS added to 0·5–1 ml of packed labelled cells, at room temperature to restore isotonicity. Final haematocrit was adjusted to about 60% and labelled cells were stored at 4 °C for a maximum of 2 days. Prior to I.V. injection into an animal, labelled cells were either suspended for 1·5–2 h in excess normal saline, resulting in control labelled cells (control LRBCs), or in saline containing 1 mg ml^{-1} hexadimethrine (HDM) in order to reduce the net negative surface charge of the labelled cells (HDM-LRBCs) by masking anionic sites. All chemicals were obtained from Sigma Chemical Co.

Effect of HDM treatment on surface charge of labelled cells

To verify whether HDM treatment reduced the net negative charge of labelled cells compared with normal RBCs and control LRBCs, anionic sites at cell surfaces were visualized by transmission electron microscopy as described by Skutelsky, Rudich & Danon (1975). Blood from a male golden hamster was collected by heart puncture and washed three times in excess PBS.

Washed cells were divided into two groups. The first group was suspended in PBS without further treatment (normal RBCs). The second group was labelled with calcein (see previous section) and further subdivided into two groups. One of these groups received no further treatment and is referred to as control LRBCs. The other group received HDM treatment and is referred to as HDM-LRBCs.

All cells were incubated with 0·5 mg ml^{-1} cationized ferritin (CF) in PBS for 15 min at room temperature and then washed and centrifuged twice in excess PBS. Five to six volumes of fixative containing 1% formaldehyde and 3% glutaraldehyde in 0·1 M phosphate buffer were added to each group of suspended cells. After fixation for 1 h at room temperature, the cells were washed twice with distilled H_2O. They were then postfixed for 1 h at 4 °C in 1% OsO_4 in PBS, washed twice in PBS, dehydrated in graded ethanol, and embedded in Epon. Sections with a thickness of 100 nm were obtained with a microtome (Richert Ultracut), mounted on copper grids and coated with carbon. A Philips EM420 transmission electron microscope was used at 80 kV.

For the sake of comparison, five cells of each type were photographed at a final magnification of × 66 000. To count ferritin particles, two more photographs were taken per cell at final magnification of × 108 000. All cationized ferritin particles on the RBC surfaces, visible on the 2-D projected micrographs of the 100 nm coupes, were counted and expressed as the number of CF particles per micrometre of (projected) RBC surface.

Effect of treatments on cell filterability

To test for differences between normal RBCs and control LRBCs and/or HDM-LRBCs with respect to the mechanical properties of the cells, the filterability of the three cell types was determined with a cell transit time analyser (CTTA). Blood was collected from three male golden hamsters and treated as described in the section 'Labelling of red blood cells'. Technical details of the CTTA have been described by others (Koutsouris *et al.* 1988*a*,*b*; Koutsouris, Guillet, Wenby & Meiselman, 1988*c*; Rendell *et al.* 1992). In short, transit times of individual cells through cylindrical pores with diameters of 5 μm are determined by measurement of the change in electrical conductivity caused by the passage of an individual cell through a pore with a length of 15 μm. The duration of the conductivity change reflects the time required for the cell to traverse the length of the pore under a known driving pressure. The set-up consists of two reservoirs separated by a polycarbonate

filter. One reservoir contains cells suspended in conducting PBS, the other is filled with PBS without cells. Electrodes are placed on both sides of the polycarbonate filter. The filter contains about thirty pores and the flow of cells through the filter was obtained at 37 °C and a driving pressure of 4 cmH$_2$O due to a fluid level difference between the suspension and the buffer. Transit times of at least 4000 cells per sample were determined and distributions of cell transit times for each sample were generated on computer printouts by numeric conversion of electrode signals by a computer. Geometric means of the transit time distributions of the samples were determined and statistically analysed (see below).

Measurements on capillary perfusion

Velocity–flux relationships of control LRBCs (8 capillaries from 3 animals) and HDM-LRBCs (12 capillaries from 3 animals) were compared with velocity–flux relationships of normal RBCs in the same capillaries. Relationships for normal RBCs and for labelled cells were successively measured after switching to the appropriate microscope objective and video camera. All measurements were performed during maximal vasodilatation by adding 10^{-4} M adenosine to the suffusate of the hamster cremaster muscle.

Cell velocity. Capillary red blood cell velocity (V, μm s^{-1}) was calculated from the time required for a cell to travel a measured segment length of vessel between two fixed points. Measured lengths were between 80 and 110 μm. Mean red blood cell velocity was obtained by averaging the velocities of five to ten cells.

Cell flux. Capillary red blood cell flux (F, s^{-1}) was measured by determining the time required for at least fifty cells to enter a capillary segment for a maximum of 5 min. If the number of cells entering the capillary was less than fifty at 5 min, this number was used to calculate the flux of cells in the 5 min period.

Cell velocity–flux relationships. Cell velocity in individual capillary segments was manipulated by partial obstruction of either the feeding arteriole or collecting venule by positioning a glass micropipette (o.d. 10 μm, closed tip) on top of the vessels. The micropipette was positioned using a mechanical micromanipulator (model MN-151; Narishige). The protocol started with the recording of cell fluxes and velocities without arteriolar or venular obstruction for 5 min. Then cell velocity was lowered by partial obstruction of the arteriole or venule. After 5 min, the obstruction was increased or decreased to manipulate red cell velocity. This

Figure 1. Cell transit time analysis (CTTA)

Box plots of the transit time distributions for normal RBCs (left), control LRBCs (centre) and HDM-LRBCs (right) flowing through cylindrical pores with a diameter of 5 μm. The horizontal lines of the 3 boxes reflect percentiles as demonstrated in the upper left corner of the figure. Median values (50% percentile) of the transit time distributions are not significantly different from the geometric means, which were 0·81, 0·78 and 0·79 ms for normal RBCs, control LRBCs and HDM-LRBCs, respectively.
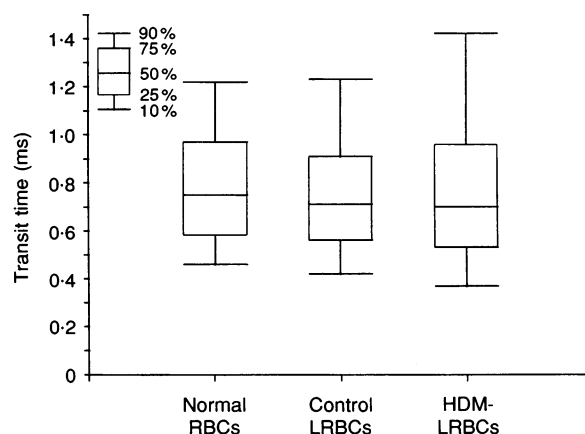
protocol was repeated until cell flux and velocity measurements were obtained over a wide range of cell velocity values.

Statistics

Data are presented as means $\pm$ s.e.m. Group means were compared using paired t tests. Significance was assessed at the 95% confidence level.

RESULTS

Cell transit time analysis

Box plots of the pooled transit time distributions for normal RBCs (left), control LRBCs (centre) and HDM-LRBCs (right) are depicted in Fig. 1. Each distribution contains data from three animals, each animal contributing to all three distributions. Comparison of geometric means of transit times of individual samples did not reveal
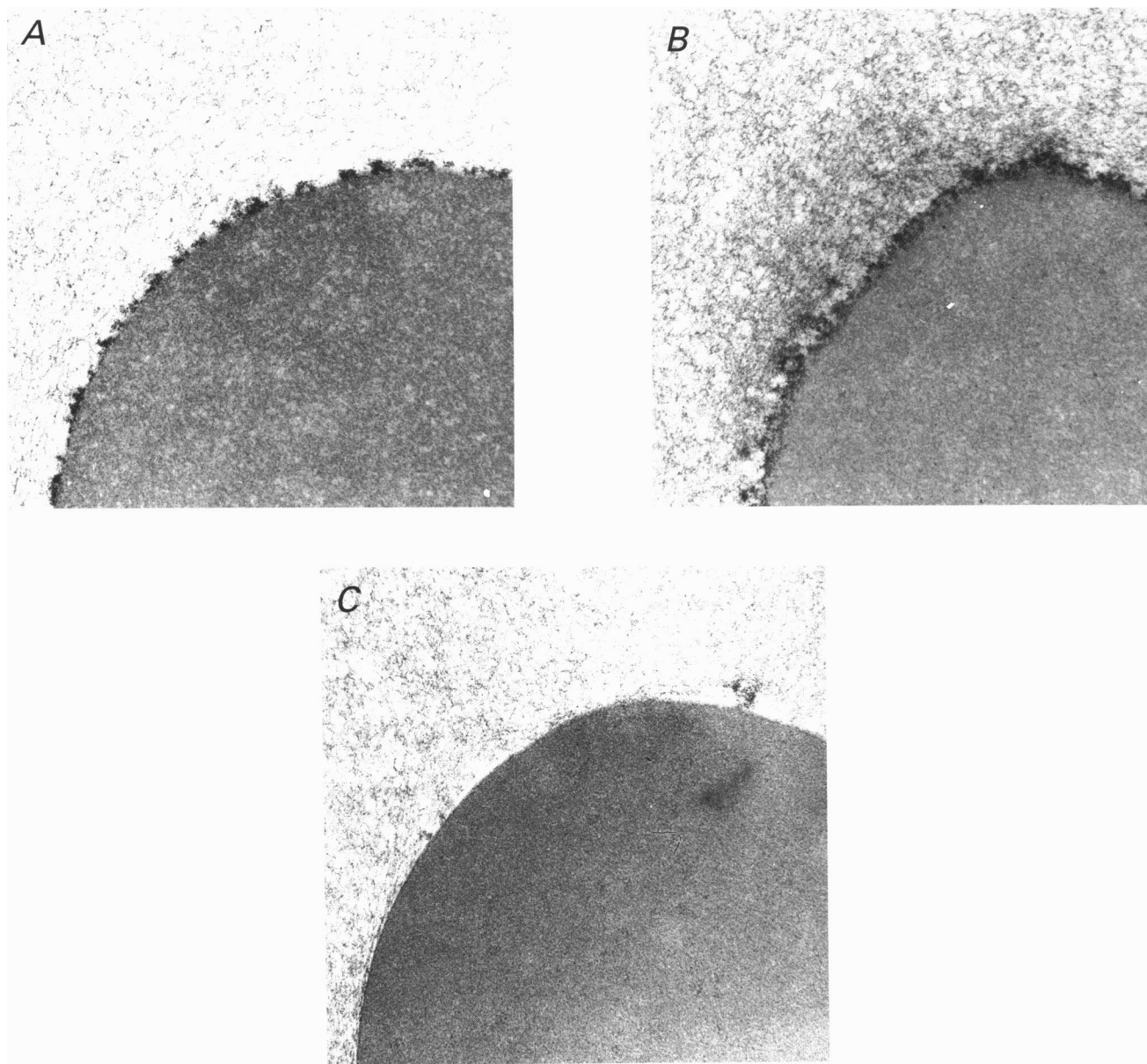
Figure 2. Reduction of CF distribution in HDM-LRBCs

Examples of transmission electron micrographs of normal RBCs (*A*), control LRBCs (*B*) and HDM-LRBCs (*C*). The pictures show deposition of cationized ferritin (CF) on the surface of the cells as a marker for the distribution of anionic sites. It is clear that CF deposition is significantly reduced for HDM-LRBCs compared with the other cell types.
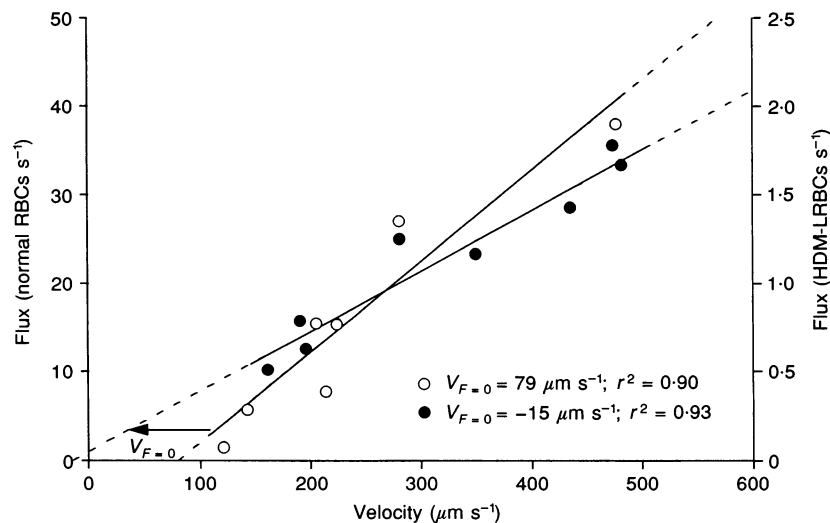
Figure 3. Capillary cell velocity–flux relationships

Example of velocity–flux relationships of normal RBCs (O) and HDM-LRBCs (●) flowing through the same capillary. $V_{F=0}$ values were 79 and -15 μm s^{-1} for normal RBCs and HDM-LRBCs, respectively.

significant differences between the three types of cells. Mean transit times were 0.81 ± 0.05, 0.78 ± 0.03 and 0.79 ± 0.03 ms for normal RBCs, control LRBCs and HDM-LRBCs, respectively.

Labelling of cell surfaces with cationized ferritin

Examples of transmission electron micrographs are depicted in Fig. 2. Mean numbers of cationic ferritin particles per micrometre of cell surface are 745 ± 168, 888 ± 321 and 158 ± 71 for normal RBCs ($n = 5$), control LRBCs ($n = 5$) and HDM-LRBCs ($n = 5$), respectively. The density of CF particles on HDM-LRBCs was significantly less than the CF density of normal RBCs and control LRBCs. No significant differences were observed between the CF densities of normal RBCs and control LRBCs. Densities of cationized ferritin of both types of labelled cells are depicted in Fig. 4 (right bars), normalized to the mean CF density of normal RBCs.

Capillary velocity–flux relationships

Examples of capillary velocity–flux relationships for normal RBCs and HDM-LRBCs measured in the same capillaries are depicted in Fig. 3. Note the difference in scale between the left and right vertical axis for the flux of normal and treated red cells. The scale difference indicates that the flux of labelled red cells was about 5% of that of normal red cells. Note that velocity and flux of HDM-treated red cells and normal red cells illustrated in this figure were measured in the same capillary. Velocity–flux relationships are linear and intercept the velocity axis at a finite value, $V_{F=0}$. It was difficult to obtain data close to $V_{F=0}$ of the slowest cells, the untreated ones, since capillary red cell motion came to a complete halt and no more red cells entered a capillary. The difference in velocities between the two types of cells close to $V_{F=0}$ is possible because of the low capillary haematocrit in that velocity range.

Figure 4. Effect of treatment on $V_{F=0}$ and CF density

Mean $V_{F=0}$ values and CF densities for HDM-LRBCs and control LRBCs, normalized to the average values of normal RBCs. Both $V_{F=0}$ values and CF densities of HDM-LRBCs, but not of control LRBCs, were significantly reduced compared with the values of normal RBCs. □, $V_{F=0}$; ▨, CF density.
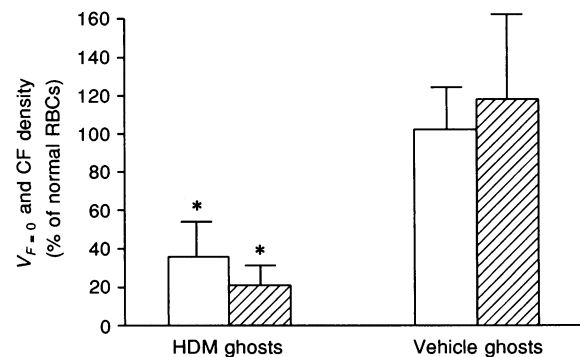
No significant differences were found when $V_{F=0}$ values for normal RBCs and control LRBCs entering the same capillaries were compared ($n = 8$). $V_{F=0}$ values were $63 \pm 14\ \mu\mathrm{m\ s^{-1}}$ for normal RBCs and $65 \pm 13\ \mu\mathrm{m\ s^{-1}}$ for control LRBCs. Squared correlation coefficients of capillary red cell velocity–flux relationships were 0.93 ± 0.02 and 0.79 ± 0.09 for normal RBCs and control LRBCs, respectively ($n = 8$).

In contrast, $V_{F=0}$ values for HDM-LRBCs and normal RBCs in the same capillaries were $31 \pm 17\ \mu\mathrm{m\ s^{-1}}$ and $86 \pm 15\ \mu\mathrm{m\ s^{-1}}$, respectively ($P < 0.05$; $n = 12$). The squared correlation coefficients of the capillary red cell velocity–flux relationships were 0.89 ± 0.04 and 0.89 ± 0.04 for normal RBCs and HDM-LRBCs, respectively ($n = 12$).

Mean $V_{F=0}$ values of control LRBCs and HDM-LRBCs, normalized to the $V_{F=0}$ values of normal RBCs, which were measured in the same capillaries, are shown in Fig. 4.

DISCUSSION

The present study demonstrates that the $V_{F=0}$ values, or yield velocities, for red blood cells entering and moving through capillary segments are decreased by a reduction in the net surface charge of RBCs, which is achieved by HDM polycation treatment. $V_{F=0}$ values for HDM-LRBCs were $31\ \mu\mathrm{m\ s^{-1}}$, which is significantly lower than the $V_{F=0}$ values for normal RBCs moving through the same capillaries ($86\ \mu\mathrm{m\ s^{-1}}$). Hence, the data presented in this study imply that RBC charge may be an important determinant of capillary blood flow, possibly because of electrostatic interactions with negatively charged components of the capillary wall and/or negatively charged plasma proteins associated with capillary endothelial cells. The reduced net surface charge of HDM-LRBCs may have eased their entry into capillaries due to reduced electrostatic repulsive forces at the capillary entrance. In addition, the flow resistance within capillaries may have been lowered for HDM-LRBCs as a result of reduced electrostatic repulsive forces between HDM-LRBCs and negatively charged capillary endothelial cell surfaces at sites of irregular capillary cross-sectional shape.

On the other hand, one could argue that reduction of the net negative charge of RBCs should slow them down inside capillaries due to increased adhesion to the capillary wall. However, a combination of trans- and epi-illumination, which allows simultaneous observation of HDM-LRBCs and normal RBCs in several capillaries with low tube haematocrit, revealed that intracapillary flow velocities of HDM-LRBCs were equal to or sometimes even higher than velocities of normal RBCs when both cell types were flowing through the same capillary at the same time, arguing against increased adhesion of HDM-LRBCs to the capillary wall. Thus, reduction of the net surface charge of

RBCs may lower cell flow resistance at the capillary entrance and within capillaries by reducing electrostatic-repulsive forces without significantly affecting the lubricating properties of the net negatively charged glycocalyx and/or associated plasma macromolecules on the luminal surface of capillary endothelial cells which shield RBCs from positively charged adhesive sites on the capillary wall.

The only parameter to be deduced from the capillary red cell velocity–flux relationships was the intercept of the velocity axis, or $V_{F=0}$, which represents a yield velocity for red blood cells entering and moving through a capillary. $V_{F=0}$ values are only obtainable by extrapolation. When red blood cell velocity was decreased close to $V_{F=0}$, red cell motion inside the capillaries would suddenly become intermittent, or the red cells would come to a complete halt and no more red cells would flow into the capillary. The flow behaviour of normal and treated RBCs was studied while both types of cells were flowing through the same capillaries. Consequently, when normal RBC flow stopped, treated RBCs stopped too and since the $V_{F=0}$ values for HDM-LRBCs were generally lower than those for normal RBCs their velocity–flux relationships had to be extrapolated over a longer trajectory. Unfortunately, the slopes of capillary velocity–flux relationships for normal and treated RBCs could not be compared as the number of injected treated cells relative to the number of circulating normal cells was not determined. However, an estimate of the slopes of the velocity–flux relationships for HDM-LRBCs relative to the slopes of velocity–flux relationships for normal RBCs can be made by assuming that the relative number of HDM-LRBCs in the circulation is equal to the ratio of the flux of HDM-LRBCs to the flux of normal RBCs at high cell velocities. The estimated fraction of HDM-LRBCs in the circulation can then be used to normalize the velocity–flux relationships of HDM-LRBCs to the velocity–flux relationships of normal RBCs. Consequently, as $V_{F=0}$ values for HDM-LRBCs are lower than those for normal RBCs, the slopes of the velocity–flux relationships for HDM-LRBCs would be less steep than the slopes of the velocity–flux relationships for normal RBCs. One explanation for this is that on lowering RBC velocities, the reduction in cell flux is more pronounced for normal cells than for HDM-LRBCs. This would mean that at identical cell velocities the normalized flux of HDM-LRBCs is always higher or equal to the flux of normal RBCs. The ratio of RBC flux to velocity is generally used to calculate the density of RBC in capillaries, which is proportional to capillary tube haematocrit. Higher fluxes of HDM-LRBCs at identical RBC velocities would thus correspond to higher capillary tube haematocrits, independent of RBC velocity. This would be consistent with the conclusion that HDM treatment reduced the resistance for red cells to enter capillaries and to flow through them at a certain capillary RBC flow velocity.

In our analysis we have assumed that the flow behaviour of HDM-LRBCs and control LRBCs is independent of that of the normal RBCs. One may wonder whether this is the case. Certainly, the overall haemodynamics of capillary blood flow must be dominated by the normal RBCs because by number they exceed the treated RBCs. However, the very fact that HDM-LRBCs behaved differently from both control LRBCs and normal RBCs demonstrates that the properties of individual RBCs must contribute significantly to the haemodynamics of blood at the microcirculatory level, in spite of supposedly unaltered bulk blood flow characteristics.

Effect of HDM polycations on RBC deformability and charge

The goal of the present study was to test whether modification of red cell charge affects capillary RBC perfusion independently of the mechanical properties of cells. RBC deformability may affect capillary blood flow because of its effect on whole blood viscosity and the deformational energy which is necessary to squeeze RBCs into capillaries (Lipowsky, Cram, Justice & Eppihimer, 1993). Stiffer red blood cells are less easily deformed, which implies that the volume flow of these cells on entering small capillary vessels may be reduced compared with the volume flow of plasma, a phenomenon also known as red cell screening. Any change of the rheological properties of red blood cells may therefore significantly alter the flux of the cells into a capillary at a certain cell velocity, and may thus affect capillary red cell velocity–flux relationships. In our experiments, RBC surface charge was reduced by treatment of the cells with hexadimethrine (HDM) polycations and these cells were filled with a fluorescent dye in order to distinguish them from normal RBCs flowing through the same capillaries. However, our measurements of the filterability of normal RBCs and of fluorescent cells with or without HDM treatment did not reveal any difference in deformability between the cell types. This finding is in agreement with the recent finding of others (Izumida *et al.* 1991) that cell filterability is not affected by treatment of RBCs with neuraminidase to reduce the net negative surface charge. However, the process of RBC surface charge reduction by neuraminidase is fundamentally different from that used in the present study: neuraminidase reduces RBC surface charge by removal of anionic sites from the cell surface, while HDM treatment reduces the charge by masking anionic sites instead of removing them. Therefore, care must be taken in comparing these two methods of reducing the net negative surface charge of RBCs. Nevertheless, we feel safe in concluding that the observed decreases in $V_{F=0}$ values for HDM-LRBCs were not due to a change of red cell filterability.

Numerous studies (Hunsicker, Shearer & Shaffer, 1981; Vehaskari, Chang, Stevens & Robson, 1984; Bertolatus & Hunsicker, 1985, 1987; Bertolatus & Klinzman, 1991; Singh, Kasinath & Lewis, 1992) report that HDM polycations can be used to neutralize negative charges on cell surfaces: they are thought to neutralize negatively charged carboxylate (COO^-) groups which are abundant on most cell types (Bertolatus & Hunsicker, 1985; Bertolatus & Klinzman, 1991). As the negative charge of RBCs is mainly due to COO^- groups (Izumida *et al.* 1991), it is likely that HDM treatment reduced the cell surface charge. However, the effect of HDM polycation treatment on RBC charge has hitherto never been explicitly demonstrated. Hence, it was necessary for us to test whether HDM treatment could, indeed, reduce the net negative charge of RBCs. In the present study, we visualized anionic sites on the RBC surface as described previously by Skutelsky *et al.* (1975): cationized ferritin (CF) particles were used as markers for anionic sites and the deposition of CF particles on the RBC surfaces was visualized with transmission electron microscopy. Although only a small number of RBCs were analysed in this way, HDM treatment clearly produced a marked reduction in the density of CF particles compared with the CF density on normal RBC surfaces. Filling of RBCs with calcein dye without further HDM treatment did not affect CF deposition (Figs 2 and 4). Thus, these findings confirm that HDM treatment can reduce the net negative surface charge of RBCs.

Possible mechanisms by which RBC charge could affect capillary red cell perfusion

The haemodynamics of microvascular blood flow have been studied extensively for several decades. It is well accepted that the distribution of red blood cells in the microcirculation is affected by the relative blood flows in daughter and parent vessels, by RBC deformability, and by the radial position and discharge haematocrit of RBCs in feeding parent vessels (Cokelet, 1982; Pries, Ley & Gaehtgens, 1986; Goldsmith, Cokelet & Gaehtgens, 1989; Pries, Ley, Claasen & Gaehtgens, 1989; Pries, Secomb, Gaehtgens & Gross, 1990).

It is unlikely that differences occurred in the daughter-to-parent flow ratios for normal and HDM-treated RBCs in the present study, as both types of cells were observed within minutes of each other, while flowing through the same capillaries in a maximally vasodilated vascular bed. Although, as discussed above, no differences in filterability of normal RBCs and HDM-LRBCs were detected in the present study, it cannot be entirely excluded that either the dye or the HDM polycation treatment resulted in deformability changes which were too small to be detected by measurement of RBC filterability. However, one would expect that if there had been a change, our treatment would have reduced deformability and so increased yield velocity. The opposite was found. Also, alterations in internal red cell viscosity produced by the reduced haemoglobin content are not dominant in the

determination of $V_{F=0}$ because no difference was found between normal and control LRBCs. We cannot rule out the possibility that the radial position of HDM-LRBCs differed in the supplying vessels from the position of normal RBCs and control LRBCs. Since RBC charge is important in counteracting cell aggregation (Izumida *et al.* 1991), the position of the HDM-LRBCs in the feeding vessels may well have been different due to altered interactions with other RBCs or with plasma macromolecules. But in this case also, one would have expected that a reduced charge would have resulted in an increased tendency to aggregate and thereby an increase in yield velocity. Furthermore, while flowing through the capillaries, HDM-LRBCs did not adhere to other red cells or to the endothelial cells lining the capillary wall which argues against a significantly increased tendency for HDM-LRBCs to aggregate.

Alternatively, it has been suggested that interactions between RBCs and the capillary endothelial glycocalyx and/or the plasma macromolecules that are associated with the capillary wall affect capillary blood flow (Klitzman & Duling, 1979; Witte, 1983, 1988; Desjardins & Duling, 1987, 1990; Duling & Desjardins, 1987). In support of this possibility, it has been demonstrated that enzymatic treatment of capillary endothelial cells *in vivo* in order to remove highly negatively charged heparan sulphate glycosaminoglycans significantly increased the flux of red cells into capillaries independently of red cell velocity (Desjardins & Duling, 1990). Further support was provided by the finding of a significant accumulation of fibrinogen and fibronectin on microvascular endothelial cells *in vivo* (Witte, 1983, 1988). Since it was merely the charge of the red cells that was manipulated in our experiments, an effect on interactions between the red cells and the glycocalyx and/or associated plasma macromolecules is at present the best working hypothesis for the interpretation of our results. Reduced electrostatic repulsion may cause red cells to enter capillaries more easily. Additionally, red cells may move more freely within capillaries with less repulsion from the capillary wall, at sites of irregular capillary cross-sectional shape without affecting the lubricating properties of the capillary endothelial cell glycocalyx.

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